In-vitro and in-vivo nematicidal activities of the cyclic dodecapeptide omphalotin A

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Abstract: Omphalotin A, a cyclic dodecapeptide produced by submerged cultures of the basidiomycete *Omphalotus olearius*, exhibited in-vitro and in-vivo nematicidal activity. *Meloidogyne incognita* was the most sensitive nematode. At 2.0 mg litre^{-1}, 50% of the nematodes were dead after one hour. *Heterodera schachtii*, *Radopholus similis* and *Pratylenchus penetrans* were affected at higher concentrations. Incorporated into agar, the compound prevented infection of cucumber seedlings by *M. incognita* at concentrations of 1 mg litre^{-1} and higher. In glasshouse tests, complete protection of cucumbers and lettuce was achieved between 2.5 and 10 mg litre^{-1}. No insecticidal activity was observed when *Plutella xylostella*, *Phaedon cochleariae* or *Spodoptera frugiperda* were fed material containing 4 g kg^{-1} of omphalotin A.

Keywords: omphalotin A; cyclic peptide; nematicidal activity; *Meloidogyne incognita*

1 INTRODUCTION

Among microbial secondary metabolites, those with nematicidal activity are comparatively rare.[1] The avermectins and milbemycins of bacterial origin are the most prominent natural helminthicides and their discovery has stimulated research in this field immensely.[2,3] Fungi as sources of nematicidal metabolites have long been neglected.[4] Omphalotin A has recently been detected in submerged cultures of the basidiomycetes *Omphalotus olearius* (de Candolle ex Fr) Singer and *Lampteromyces japonicus* (Kawamura) Singer.[5,6] It is a cyclic dodecapeptide and thus the largest member of the group of fungal cyclic peptides to which the immunosuppressive cyclosporins also belong.[7] Its structure is shown in Fig 1. Whereas cyclosporin A was almost inactive, omphalotin A showed a remarkable selective nematicidal activity towards *Meloidogyne incognita* (Kofoid & White) Chitwood which is inhibited *in vitro* at 0.75 mg litre^{-1}, whereas the saprophytic *Caenorhabditis elegans* Maupas is affected only at 50-fold higher concentrations.[8,9] Omphalotin A showed no or very low cytotoxicity, and no phytotoxicity against *Setaria italica* (L.) P.B. and *Lepidium sativum* L.[8] The compound had no effect on Gram-positive or Gram-negative bacteria and no antifungal activity against *Mucor miehei* Cooney & Emerson, *Paecilomyces varorii* Bainier, *Nematospora coryli* Peglion, *Ustilago nuda* (Jensen) Rostrup, *Rhodotorula glutinis* (Fr) Harrison or *Saccharomyces cerevisiae* Meyer ex Hansen. This interesting spectrum of activity prompted the testing of additional nematodes and the evaluation of the insecticidal and plant protective activities of omphalotin A in glasshouse trials.

In this paper, the in-vitro nematicidal and insecticidal activities of omphalotin A as well as its in-vivo effects on *M. incognita* are reported.

2 METHODS

2.1 Nematicidal activity

2.1.1 Meloidogyne incognita

The microtiter plate assay has been described before.[5] Two hundred and fifty nematodes ml^{-1} were used.

Assay for plant protectant activity *in vitro:* omphalotin A, dissolved in methanol, was added to Gamborg's B5 agar after sterilization of the agar medium. The agar was poured into agar plates (5.5 cm diameter) and one cucumber seed (*Cucumis sativus* L. cv. Hoffman's Giganta) which had been

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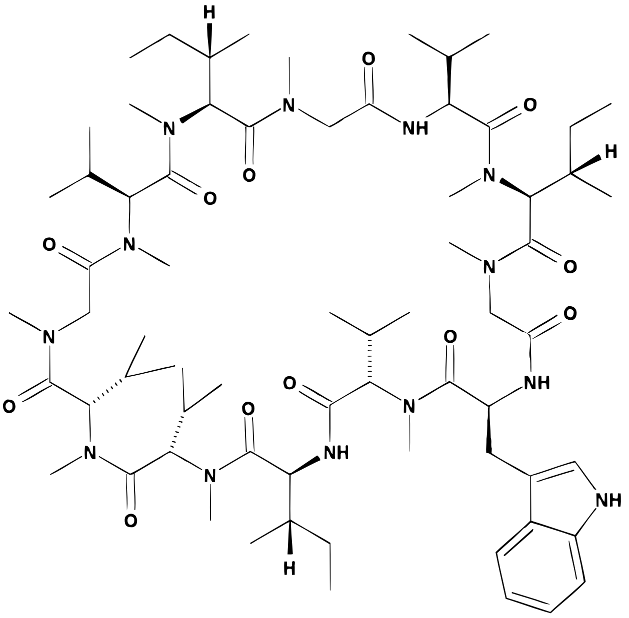

Figure 1. Structure of omphalotin A.

surface sterilized with NaOCl (20 g litre^{-1} in water) for 30 min was placed on the agar. The plates were incubated at 27°C in the dark. After two weeks, 500 axenically prepared second-stage larvae of *M. incognita* were placed next to the roots of the seedling and the incubation continued for three months in the dark. Every two weeks the seedlings were evaluated for signs of disease. The test was carried out three times in duplicate.

For in-vivo experiments *C. sativus* L. cv. Bella F1 was grown in a soil and leaf litter mixture. Two plants, 14 days old, in one pot (7 × 7 × 6 cm) were treated with water (10 ml) containing omphalotin and then 500 nematodes (L2 stage) were added. The pots were left in the glasshouse at 26°C during the day and 20°C during the night. After three weeks the disease symptoms of the roots were evaluated by comparing the number of galls with water-treated control plants. As a positive control, fenamiphos was used.

For assays with lettuce (*Lactuca sativa* L. var. *capitata* L.), soil (250 ml) was mixed with water (10 ml) containing omphalotin A and 700 nematodes. The mixture was poured into pots (7 × 7 × 6 cm). Seeds were put onto the soil and covered with silica sand. The pots were kept humid and were incubated in the glasshouse at 25°C for 24 days. For the evaluation, the roots were washed, the galls counted and their number compared with the control without omphalotin A.

2.1.2 *Other nematodes*

Heterodera schachtii Schmidt, *Caenorhabditis elegans*, *Radopholus similis* (Cobb) Thorne and *Pratylenchus penetrans* (Cobb) Filipjev & Stekhoven were tested in 24-well plates. Omphalotin A was dissolved in methanol and added to the wells. After evaporation of the solvent, sodium chloride solution (0.8 ml; 9 g litre^{-1}) and 100 nematodes in water (0.2 ml) were added. The plates were incubated under gentle shaking at 22°C. After 24 h, the percentage of dead nematodes was evaluated according to Abbott.[10]

2.2 Insecticidal activity

Plutella xylostella L. and *Phaedon cochleariae* F. were cultured on savoy cabbage (*Brassica oleracea* L. var. *sabauda* L.), *Spodoptera frugiperda* J.E. Smith on corn plants (*Zea mays* L.).

For insecticidal assays, omphalotin A dissolved in methanol was spotted onto plant leaf disc (10 mm diameter). After evaporation of the solvent, two discs were placed in a Petri dish (55 mm diameter) together with two larvae (third-stage) and a water-saturated filter paper to maintain the humidity. After 48 h, an untreated leaf was added. After 72 h, the mortality rate was compared with the methanol-treated control. The tests were carried out in duplicate and repeated three times. *S. frugiperda* larvae (third-stage) were tested with corn leaves (1.5 × 2 cm) and only one larva per Petri dish. The tests were carried out with four larvae and repeated three times.

2.3 Chemicals

Gamborg's B5 plant powder was purchased from Serva, Heidelberg, Bacto-agar from Difco, Detroit. Omphalotin A was isolated from submerged cultures of *Omphalotus olearius* TA 90170 as described earlier.[7] Other chemicals (p.a. quality) were from Merck, Darmstadt.

3 RESULTS AND DISCUSSION

The spectrum of nematicidal activity is given in Table 1. Among the tested nematodes, *M. incognita* was the most sensitive organism. *H. schachtii* and *P. penetrans* were also affected but were at least one order of magnitude less sensitive. The sensitivity of *R. similis* was similar to that of *C. elegans*. The in-vitro plant-protectant activity of omphalotin A was remarkable, as can be seen in Table 2. At concentrations as low as 0.1 μg ml^{-1}, the number of galls was reduced and at concentrations higher than 1 μg ml^{-1} no galls were seen. The cucumber seedlings were not impaired by 4 μg ml^{-1}, the highest concentration tested. Seedlings of *L. sativa* were not affected when incubated in water containing 100 μg ml^{-1}. This is in agreement with our previous finding that omphalotin A was not phytotoxic to *Lepidium sativum* and *Setaria italica*.[8] Glasshouse trials with *L. sativa* and *C. sativus* also revealed good plant-protectant activity towards *M. incognita*. The results are given in Table 3. Complete protection of *L. sativa* can be achieved by 2.5 mg litre^{-1} of omphalotin. Concentrations needed for the protection of cucumbers are somewhat higher.

Table 1. Nematicidal activity *in vitro* of omphalotin A against phytopathogenic and saprophytic nematodes

Nematode	Nematicidal activity of omphalotin A			
	Mortality (%)			LD_{50}[a] ($mg\ litre^{-1}$)
	5 mg litre^{-1}	10 mg litre^{-1}	30 mg litre^{-1}	
M. incognita	100	100	100	2.0
C. elegans	<50	<50	60	> 75.0
H. schachtii	40	60	85	30
R. similis	not tested	46	54	>75
P. penetrans	not tested	67	97	25

[a] LD_{50}: Lethal dose for 50% of the nematodes after 1 h

Insecticidal activities towards *P. xylostella*, *P. cochleariae* or *S. frugiperda* could not be detected when the animals were fed with plant material containing 4.0 g kg^{-1}.

While cyclic depsipeptides with insecticidal activity are known from many fungi, especially insect pathogenic species,[11] those with nematicidal activities are rare, PF 1022A isolated from *Mycelia sterilia* being one example.[12] Omphalotin A is the first cyclic dodecapeptide isolated from fungal cultures. Though it is structurally related to the cyclosporins, the modes of action of the two peptides seem to be different, since cyclosporin A exhibits only very low nematicidal activity towards *M. incognita*.[2] Whether omphalotin A has immunosuppressive activity remains to be elucidated. While cyclosporin A contains D-alanine and an uncommon amino acid, and shows insecticidal activity towards mosquito larvae,[13] the configuration of the amino acids in omphalotin A is not yet known. It has been shown for PF 1022A that the configuration of the amino acids plays an essential role, its synthetic enantiomer PF 1022-001 being devoid of anthelminitic activity.[14,15] Cyclic peptides have rarely been reported from mycelia of basidiomycetes, though fruiting bodies of *Amanita*, *Gallerina* and *Lepiota* species contain amatoxins and phallotoxins, bicyclic hepta- and octapeptides.[16] In mycelial cultures, however, only *A. verna* was found to produce amatoxins.[17]

The high susceptibility of *M. incognita* towards omphalotin A might lead to a hitherto unknown target site in this plant-pathogenic nematode. Therefore, the elucidation of the mode of action is highly desirable. Unfortunately, at present the low yield of omphalotin A hampers broad studies. In addition, genetic manipulations in order to increase its yield are difficult with dikaryotic mycelia of basidiomycetes.

Table 2. In-vitro plant protectant activity of omphalotin A

Concentration ($mg\ litre^{-1}$)	Galls compared to the control (%)
0	100
0.1	33
0.25	23
0.5	20
0.75	14
1.0	0

One cucumber seedling on an agar plate was inoculated with 500 larvae of *Meloidogyne incognita*.

Table 3. Plant protectant activity of omphalotin A in glasshouse trials with *Lactuca sativa* and *Cucumis sativus*

Omphalotin ($mg\ litre^{-1}$)[a]	Protectant activity (%)	
	Lactuca sativa	*Cucumis sativus*
0.5	48	not tested
2.5	96	54
5.0	99	79
10.0	98	96

[a] The concentration of omphalotin A is calculated per volume of soil; application of fenamiphos at 0.3 mg litre^{-1} resulted in total protection of cucumber and 4 mg litre^{-1} of lettuce

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